

Satisfiability, sequence niches, and molecular codes in cellular signaling

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Biological information processing as implemented by regulatory and signaling networks in living cells requires sufficient specificity of molecular interaction to distinguish signals from one another, but much of regulation and signaling involves somewhat fuzzy and promiscuous recognition of molecular sequences and structures, which can leave systems vulnerable to crosstalk. This paper examines a simple computational model of protein-protein interactions which reveals both a sharp onset of crosstalk and a fragmentation of the neutral network of viable solutions as more proteins compete for regions of sequence space, revealing intrinsic limits to reliable signaling in the face of promiscuity. These results suggest connections to both phase transitions in constraint satisfaction problems and coding theory bounds on the size of communication codes.

1. INTRODUCTION

The functioning of complex biomolecular pathways hinges on conveying molecular signals reliably in the stochastic and evolving milieu of living cells. These signals are mediated by molecular interactions that distinguish physiological binding partners from myriad other cellular constituents: this ability to distinguish functional signals from the molecular noise is ultimately the source of information processing in cellular networks. But molecular recognition is subtle: many of the molecular interactions involved in cellular regulatory and signaling pathways do not involve highly specific “lock and key” binding, but instead are characterized by more fuzzy and promiscuous recognition of families of sequences and configurations [1, 2, 3]. Furthermore, there are often multiple types of molecules within a cell that can bind to the same target, such as different proteins containing homologous copies of a modular interaction domain. We therefore ask a basic theoretical question concerning cellular signaling in crowded sequence spaces, where multiple proteins bind to similar families of molecular sequences and structures: under what circumstances can crosstalk be avoided in such a system? This paper investigates a simple null model, associated with random molecular sequences, that is amenable to analysis and suggests connections to recent work on phase transitions in combinatorial NP-complete problems. This random model is not directly applicable to the evolved molecular sequences found in nature, but serves as a useful first step in defining the landscape of constraint satisfaction in cellular signaling.

The theory of communication in noisy channels, dating back to the seminal work of Shannon [4, 5], also provides a useful framework in which to interpret cellular signals. Engineered error-correcting codes embed messages in higher-dimensional spaces (e.g., via encoded checks on the message integrity), to insulate each possible codeword within a sphere in the embedding space. By packing such spheres so that they are disjoint, any corrupted message word can (up to some defined number of errors) be uniquely associated with an original

code word. In molecular signaling, *sequence recognition volumes* play a similar role: these volumes describe the sets of sequences recognized (i.e., bound with significant probability) by various target molecules. In molecular signaling, overlapping recognition of sequences precludes the sort of disjoint geometries found in engineered codes. Instead of asking, therefore, whether *all* messages can be communicated through a protein signaling channel, we will focus here instead on whether *any* message can be so conveyed (under the assumption that evolutionary selection might find such a solution if it does in principle exist). Addressing the discrimination of potentially ambiguous signals, this work is related to issues arising in error-correcting codes, but geometrically it is in some ways more similar to problems involving covering codes [6] and identifying codes [7]. A central result presented here, which establishes limits on the number of proteins that can compete for regions in sequence space before crosstalk becomes likely, is akin to a bound on the size of a code in a communication system.

This work was motivated in part by experiments on SH3-mediated signaling in yeast (*Saccharomyces cerevisiae*), by Zarrinpar, Park and Lim [8]. SH3 domains constitute a family of conserved modular protein domains, known to bind to a set of proline-rich peptide sequences (the so-called “PXXP” motif, which actually consists of a larger peptide of approximately 8-10 residues)[2, 9]. Because of this interaction promiscuity, and because several proteins in yeast contain SH3 domains, it was not obvious whether there would be crosstalk among pathways involving different SH3-containing proteins. Zarrinpar *et al.* probed the yeast high-osmolarity signaling pathway, which involves the interaction of Sho1 (a protein with an SH3 domain) and Pbs2 (containing a PXXP motif). By making chimeric versions of Sho1 containing different SH3 domains, they demonstrated that none of the other native yeast SH3 domains were capable of interacting with Pbs2, but that half of the metazoan SH3 domains they tested were able to do so. They surmised that there has been an evolutionary selection against crosstalk with that pathway in yeast, with protein sequences having co-evolved such that the Pbs2 ligand lies in a niche in sequence space where it

is recognized by only the Sho1 SH3 domain. Since there has been no such selection pressure to avoid crosstalk in other organisms, the Pbs2 motif bound to non-native SH3 domains with greater probability. (See supplementary text and Figure S.1 for further discussion.) It is the structure of these sorts of *sequence niches* that form the core of this paper. In related work, Sear has computed the capability of a set of competitive protein-protein interactions, and examined crosstalk avoidance in a model motivated by the same set of yeast signaling experiments [10, 11].

The fundamental questions posed by the experiments on SH3 signaling in yeast extend beyond that particular system. A classic problem in immunology is the ability of antibodies to discriminate between “self” and “nonself” antigens, and early work addressed how large a recognition region needs to be in order to reliably perform this discrimination [12]. In gene regulation, transcription factors (TFs) that regulate gene expression by binding to DNA are organized in families that often recognize similar sorts of sequences. Recent work in that area has explored tradeoffs between binding specificity and system robustness [13], balances between selection and mutation [14], evolutionary divergence of competing TF-binding sequence pairs to avoid crosstalk [15], and the application of ideas from coding theory to understand limits on the size of TF families [16]. In bacterial signaling, the possibility of crosstalk among two-component regulatory systems, whereby multiple response regulators are activated by a single sensor kinase, has also been explored to gain insight into how environmental signals are combined [17, 18, 19].

2. RESULTS

2.1. The Sequence Niche Question

We begin by distilling the central question to be considered here: under what conditions does a unique sequence niche exist so that signaling without crosstalk might be possible? To address this question, we adopt a highly abstracted model of protein-protein interaction, in which protein sequences are represented by binary strings of length L (consisting of 0’s and 1’s) rather than as peptide strings in the 20-letter amino acid alphabet. (Binary sequence models, such as the HP model, has been used in the study of protein folding [20], although it remains an open question as to whether there is an appropriate coarse-grained alphabet capable of capturing the essential biochemistry of protein-protein interactions involved in signaling.) In this model, binding of a peptide sequence to a protein is achieved if the sequence is sufficiently close to the consensus sequence recognized by the protein, with Hamming distance used as a measure of closeness: two sequences bind if they differ in at most R positions, given some promiscuity radius R . Given this representation, we can pose the

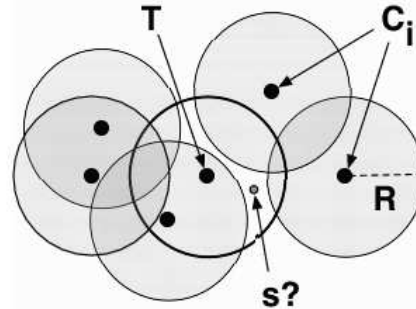


FIG. 1: The Sequence Niche Question: given a target protein sequence T and a set of N crosstalking protein sequences $\{C_i\}$, is there a sequence s that is bound by T but not by any of the proteins C_i ? In this model, sequences are binary strings of length L , and two sequences bind if the Hamming distance between them is less than or equal to R .

Sequence Niche Question, phrased and typeset in the canonical style of Garey and Johnson [21] and illustrated schematically in Fig. 1:

SEQUENCE NICHE

INSTANCE: Binary sequence T of length L , a set of binary crosstalk sequences C_i , for $i = 1, \dots, N$, each of length L , and an integer R , $0 \leq R \leq L$.

QUESTION: Is there a binary sequence s of length L such that $H(T, s) \leq R$ and $H(C_i, s) > R$ for $i = 1, \dots, N$, where $H(x, y)$ is the Hamming distance between sequences x and y ?

The Sequence Niche Question (SNQ) is a rephrasing of the Distinguishing String Selection Problem (DSSP), as defined by Lancot *et al.* [22]. (The DSSP allows more for multiple “good” strings S_c to be matched within some Hamming distance k_c and multiple “bad” strings S_f to be avoided outside some Hamming distance k_f .) The DSSP was proven to be NP-complete [22]; the SNQ is the DSSP with $S_c = 1$ and $k_c = k_f$, but the computational complexity of the DSSP does not depend on the values of these parameters, so the SNQ is also NP-complete. The SNQ is similar in spirit to the well-known computer science problem SAT (and its specialization k -SAT), in that these problems ask whether there exists a solution that satisfies a set of (potentially conflicting) constraints [21]. Borrowing from the language of SAT, we say a particular instance of the SNQ is “satisfiable” when a solution s exists, and “unsatisfiable” when there is no such solution. The SNQ asks whether discrimination of one target protein from a background of crosstalking proteins is possible. A symmetric generalization of this problem would ascertain whether every protein in a collection is distinguishable, that is, whether there is a separate sequence niche for each of the N proteins. This generalized SNQ is essentially that considered by Sear [11],

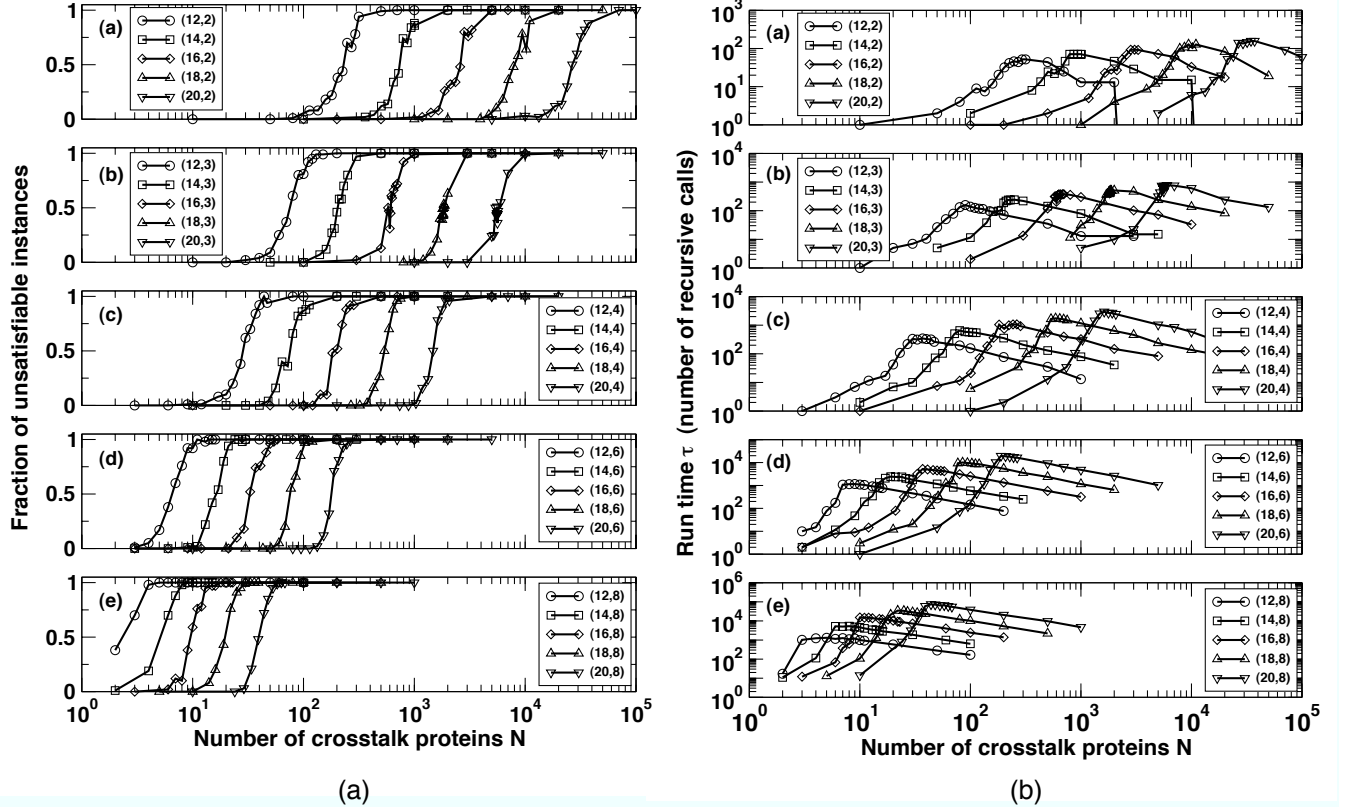


FIG. 2: (a) Average fraction of unsatisfiable instances of the random SNQ as a function of L, R , and N ((L, R) specified in figure legend, N varying along x-axis). (b) Average run time τ of the SNQ decision (number of recursive calls in the solution algorithm) for the same instances depicted in (a). Averages are over 100 instances of the SNQ for each (L, R, N) set.

although he did so for a 4-letter protein alphabet and a more realistic treatment of the binding kinetics than simple Hamming distances, demonstrating that for at least some parameters, such discrimination is possible. The generalized SNQ is presumably in the same complexity class as the single-target SNQ, since deciding it simply involves deciding N separate SNQs.

2.2. Satisfiability of the random SNQ

The NP-completeness of the SNQ is a statement about its worst-case complexity, but there has been increasing interest in recent years in quantifying the typical-case complexity of NP-hard problems. A common strategy is to examine ensembles of random instances of NP-hard problems, investigating how solution complexity depends upon parameters that characterize those random instances. A similar strategy is adopted here.

Multiple random instances of the SNQ were examined (with uniform equal probability of 0's and 1's in the sequence strings), for various values of the problem parameters L , R , and N . Figure 2(a) shows the average unsat-

isfiable fraction of random SNQ instances as a function of the number of crosstalking proteins N , averaged over an ensemble of 100 random instances for each N . In addition, Figure 2(b) shows the average run time τ required for determining whether or not an instance is satisfiable (where run time is measured in units of the number of recursive calls to the solution algorithm of Gramm *et al.* [23]). Fig. 2(a) demonstrates a transition from satisfiability (SAT) to unsatisfiability (UNSAT) as the number of crosstalking proteins is increased. Rather than a gradual diminution in the capacity for reliable signaling, the SNQ exhibits a relatively abrupt switch as $\log N$ increases. Fig. 2(b) reveals, for the same set of parameter values, that the run time of the solution algorithm reaches a maximum near the point of the SAT-UNSAT transition. In other words, it becomes significantly more difficult to decide if a given instance is satisfiable or not when that instance lies near the transition. The characteristic scales of the random SNQ are seen to vary over orders of magnitude. For the solution run times, this is perhaps not surprising: since the SNQ is NP-complete, we expect the worst-case run time of the solution algorithm to be exponential in the size of the problem.

2.3. Scaling of the SNQ transition: a satisfiability bound on the number of crosstalk proteins

Even though the characteristic scales of the SNQ vary by orders of magnitude, there is a scaling structure evident in those data. This structure is understood by considering the geometric and probabilistic nature of the random SNQ. A given instance is unsatisfiable if the target volume (i.e., the Hamming sphere of radius R surrounding the target sequence T) is completely covered by the union of the crosstalk volumes (centered about the crosstalk sequences $\{C\}$), a process that is illustrated schematically in Fig. 3(a). We can estimate the critical number of crosstalk proteins N_c needed to cover the sequence volume of the target protein (see supplementary text for full derivation):

$$N_c = \frac{\log(1/V)}{\log(1 - V/V_0)} \quad (1)$$

where $V_0(L) = 2^L$ is the total number of possible binary sequences of length L , and $V(L, R) = \sum_{n=0}^R \binom{L}{n}$ is the number of binary sequences in a ball of Hamming radius R about a given sequence. We can interpret this as a *random satisfiability bound* on the approximate number of randomly distributed proteins that can coexist without crosstalk.

With this critical protein number, we can rescale the raw satisfiability and run time data of Fig. 2. These rescaled data are shown in Fig. 3; in (b) and (c) the protein number (x-axis) is scaled as $N \rightarrow (N - N_c)/N_c$, and in (c), the run time data (y-axis) are scaled by the exponentially growing number of sequences in the search tree $V(L, R)$ that in principle need to be considered. The collapse of each set of unscaled data onto a reasonably compact scaling form suggests this simple description is correct.

2.4. Fragmentation of the solution space

Previously we considered whether there is *any* solution to a given instance of the SNQ. Here we examine the structure of the space of *all* satisfying solutions for an instance, as determined via exhaustive enumeration.

Consider a fixed target sequence T and a set of potential crosstalk sequences $\{C\}$. Imagine introducing crosstalk sequences one at a time, and identifying the set of all sequences $\{s_N\}$ that satisfy the SNQ for that instance with N crosstalk sequences. Of particular interest here is the size and structure of the solution set $\{s_N\}$ as a function of the number of proteins N . For each set, we assemble a graph whose nodes are sequences s that satisfy the SNQ and whose edges connect satisfying sequences if they are neighbors on the hypercube, i.e., if their Hamming distance from each other is 1. This graph represents the *neutral network* of all solutions to a *given* instance of the SNQ, along which single point mutations

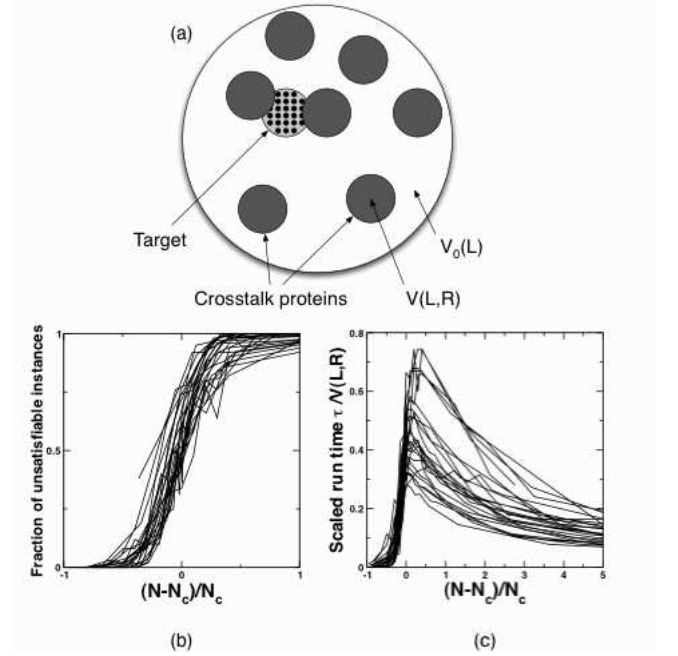


FIG. 3: Scaling description of the SAT-UNSAT transition in the SNQ. (a) Schematic depiction of the covering of available sequences (black dots) in the target volume as crosstalk proteins (gray circles) are laid down randomly. (b, c) Scaling of the satisfiability and run time data in Fig. 2 based on the scaling theory presented: (b) the number of crosstalk proteins N are scaled by $N \rightarrow (N - N_c)/N_c$, and (c) in addition to scaling N , the run times τ are scaled by the number of sequences in the target volume $V(L, R)$ that must be considered.

to the solution string (bit flips) can be made without producing crosstalk. For various N , we can compute the set of connected components of the resulting graph. The change in the structure of the neutral network of satisfying solutions is illustrated, for a given problem instance with $L = 16$ and $R = 6$, in Fig. 4. For small numbers of proteins (Fig. 4(a)), there are many possible solutions to the SNQ, and those solutions all coalesce into one connected cluster, such that any solution can be reached from any other via a succession of single-bit flips to the solution string. As N increases (Fig. 4(b)), the number of satisfying solutions decreases, and the connected cluster of solutions is fragmented into many disjoint sets (still dominated by a central core). This fragmentation and evaporation of the sequence clusters continues for larger N (Fig. 4(c)), until finally all solutions disappear, and unique signaling is no longer possible. While the neutral networks shown reveal the effects of mutations in the solution string s , it should be noted that single point mutations in the sequences representing the centers of the proteins T and $\{C\}$ can result in drastic changes in the neutral network topology, e.g., by fragmenting a single large cluster into a set of smaller ones.

A summary of these trends is shown in Fig. 4(d), by averaging over many SNQ instances (for $L = 16$ and

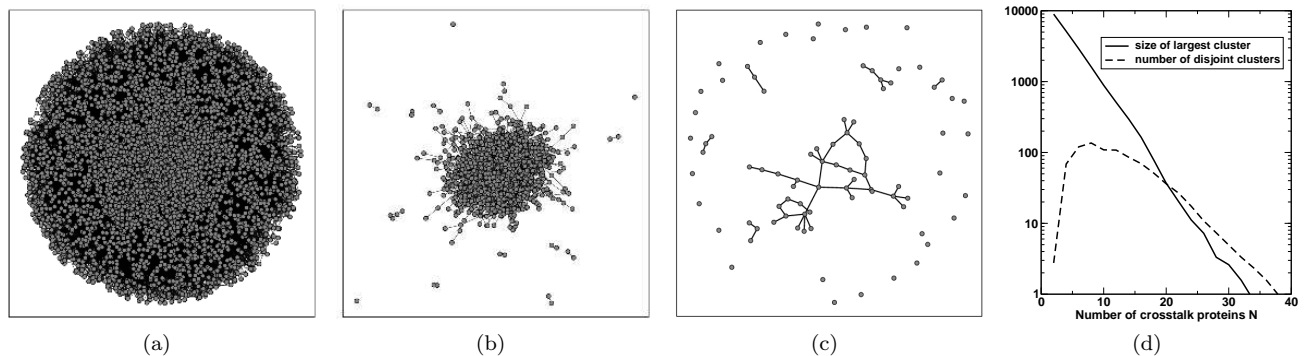


FIG. 4: Fragmentation of the solution space as the SAT-UNSAT transition is approached. (a, b, c) The neutral network of satisfying solutions $\{s_N\}$ for one particular problem instance ($L = 16, R = 6$), as a function of number of crosstalking proteins N . Satisfying sequences (nodes) are connected by edges (lines) in a network if they are separated by Hamming distance 1. (The spatial layout of nodes has no meaning; all sequences are vertices on an L -dimensional hypercube.) (a) $N = 4$: there are 5786 satisfying solutions in one large connected component. This cluster is broken up into multiple pieces as N increases. (b) $N = 12$: 1226 sequences are distributed among 18 connected components. (c) $N = 20$: only 85 sequences remain viable, scattered across 38 disjoint components. (d) For $L = 16, R = 6$, average values of the size of the largest connected sequence cluster (solid line) and the number of disjoint clusters (dashed line) as a function of N , averaged over 100 SNQ instances for each value of N .

$R = 6$). This reveals that the size (i.e., the number of nodes) of the largest cluster (solid line) decreases roughly exponentially with crosstalk number N . We can understand this decrease in part by considering the geometric argument summarized in Fig. 3(a); see the supplementary text for details. Also shown in Fig. 4(d) is the number of disjoint clusters (dashed line); this is seen to initially increase with N – as the single satisfying solution cluster is fragmented – and then decrease – as small sequence clusters evaporate in the presence of new crosstalk proteins. Fig. 4 reveals a number of isolated clusters of size 1, but these problem sizes are rather small (given the computational burdens of exhaustive enumeration). It is an open question whether nontrivial cluster size distributions will reveal themselves as larger problem sizes are considered.

3. DISCUSSION

The goal of this paper has been to examine the limitations of crosstalk-free signaling in a simple model of competitive protein-protein interactions, as a first step toward developing a more comprehensive and realistic theory. The numerical experiments presented were motivated by phase transitions observed in the random k -SAT problem [24, 25, 26, 27], where there is a SAT-UNSAT transition as the ratio of constraints to variables is increased. The numerical results presented for the SNQ demonstrate something similar: a relatively sharp transition from satisfiability to unsatisfiability with increasing competition for sequence space, along with an increase in computational complexity near the transition. Phase transitions have been studied in a number of NP-hard problems, although applications to biological problems

have been scant and generally at coarser levels of biological description [28, 29], despite significant interest in the computational complexity of problems involving sequence matching and discrimination [22, 23]. A second phase transition has more recently been identified in k -SAT, lurking near the SAT-UNSAT phase boundary, involving the fragmentation of the set of satisfying solutions [30, 31, 32]. We find evidence for such a fragmentation transition in small instances of the SNQ, although further theoretical and computational work is needed to fully characterize these transitions, which are only strictly defined in the limit of infinite system size.

Of particular interest are the possible biological implications of these results. Where, for example, are signaling systems in nature situated with respect to these types of phase boundaries, and what sorts of codes has evolution uncovered in such systems? Have evolutionary innovations – such as novel interaction domains [16], or scaffolds that localize signaling proteins [33] – arisen to rescue cellular networks from the precipice of crosstalk? Signaling interactions do not occur in isolation, but often involve compartmentalization or localization (e.g., on scaffolds) that confer context-dependent specificity in addition to the intrinsic sequence specificity addressed here [34, 35, 36]. In addition, fragmentation of the network of satisfying solutions of the sort demonstrated here leads to complex neutral network topologies. The extent to which neutral network topology influences evolution remains an open question [37, 38]. Neutral network fragmentation could lead to biological systems becoming frozen in local regions of sequence space, unable to mutate to other satisfactory configurations far away. This could produce a sort of speciation at the molecular scale, perhaps shedding light on phylogenetic relationships among related protein interaction domains. Larger-scale genomic rear-

rangements, such as homologous recombination and horizontal transfer, may play a role in helping biological communication systems become unstuck from a glassy, fragmented phase where single point mutations are unable to do so.

Examination of the SAT-UNSAT transition in random instances of the SNQ led to the derivation of a random satisfiability bound (eq. (S8)). This represents an upper limit to the number of randomly distributed sequences that can coexist without crosstalk becoming likely. While the bound was motivated by the SAT-UNSAT transition, it is also usefully interpreted within the context of coding theory bounds on the size of codes. Whereas a sphere-packing bound [5] describes the number of Hamming spheres of radius R that can be packed in L dimensions with no overlap, and a smooth coding bound [16] allows for some overlap of sequence recognition spheres, our satisfiability bound is applicable to a dense, overpacked limit when all capacity for uniquely distinguishing signals disappears. The bound presented in eq. (S8) is explicitly applicable to binary sequences without reverse-complement symmetry. It is straightforwardly generalizable (see supplementary text), within the assumption that binding is entirely dictated by the Hamming distance between two sequences, to sequences with larger alphabets (e.g., 20 amino acids) or to sequences with reverse-complement symmetry (e.g., as has been done for other code bounds treating DNA sequences [16, 39]).

Protein sequences and sequence niches involved in cellular signaling have, of course, been sculpted by evolution. We might expect evolution to be able to find better encoding schemes than the random placement considered here, by arranging sequence recognition volumes to maximize fitness. Addressing this question, however, requires consideration of several factors. First, it is not obvious what fitness measure is optimized by natural selection. If discrimination among different sequences were the only determinant of fitness, we might expect encodings to more closely resemble sphere packings, with recognition volumes maximally distinct from one another. Other determinants could alter such packings, however; a fitness advantage from some weak crosstalk, perhaps as a form of degeneracy or functional redundancy [40], might keep recognition volumes from diverging too far from one another. And it must be remembered that evolutionary mutation plays a central role in posing these constraint satisfaction problems in the first place, in that gene duplication leads to the creation of homologous proteins that recognize similar substrates. The random limit considered here, while useful for analysis, is not directly relevant to the biology of duplicated proteins that may diverge from one another just far enough to be distinguishable [15].

An examination of experimental and genomic data for model systems is an obvious next step, both to probe the structure and evolution of sequence niches in nature, and to develop more realistic and predictive models of protein-protein interaction. The experimental work re-

ported in ref. [8] included a series of single-base-pair mis-sense mutations to the native yeast Pbs2 motif, to probe the sequence space around that motif. All such mutations resulted in an increased cross-reactivity with other yeast SH3 domains, suggesting that the Pbs2 ligand lies near the periphery of a possible sparse and tenuous sequence niche, but further examination of yeast SH3 interaction data is needed to better characterize that. Fortunately, there has been considerable experimental work in screening synthetic peptide ligands to map out the sequence recognition volumes of SH3 domains in several proteins [41], and similar sorts of data are becoming available for systems such as two-component regulators [19]. Computational classifiers (e.g., weight matrices and neural networks) trained on protein-protein interaction data have been used to make predictions about binding affinities of particular proteins to arbitrary peptides [42, 43]. With a combination of experimental data, computational models, and a theoretical understanding of the complexities of constraint satisfaction problems, we can aim to map out the structure of high-dimensional sequence niches underlying cellular decision making in biological systems.

4. ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

S.1. Derivation of critical number of crosstalking proteins (random satisfiability bound)

Here we derive the result stated in eq. (1) of the main text, the critical number of crosstalking proteins N_c for a given sequence length L and promiscuity radius R , which we can interpret as a random satisfiability bound for the size of the protein-protein interaction code. A given instance of the SNQ is unsatisfiable if the target volume (i.e., the Hamming sphere of radius R surrounding the target sequence T) is completely covered by the union of the crosstalk volumes (centered about the crosstalk sequences $\{C\}$), a process that is illustrated schematically in the main text in Fig. 3(a). We can estimate the critical number of crosstalk proteins N_c needed to cover the sequence volume of the target protein. For a given binary string of length L , the number of sequences $V(L, R)$ in a ball of Hamming radius R is

$$V(L, R) = \sum_{n=0}^R \binom{L}{n} \quad (\text{S1})$$

and the total possible number of sequences $V_0(L)$ is

$$V_0(L) = 2^L \quad (\text{S2})$$

Let q be the ratio of these sequence volumes:

$$q \equiv V/V_0 \quad (\text{S3})$$

We consider depositing at random sequence volumes of size $V(L, R)$ in a space of volume $V_0(L)$. From the binomial distribution, the probability that a given point in sequence space is covered n times after N proteins have been deposited is

$$P_q(n|N) = \binom{N}{n} q^n (1-q)^{N-n} \quad (\text{S4})$$

Therefore the probability $U_q(N)$ that a given point in sequence space is left *uncovered* by N proteins is

$$U_q(N) = P_q(0|N) = (1-q)^N \quad (\text{S5})$$

We can thus estimate the average number of sequences $S_u(V, q, N)$ in the target volume V left uncovered by N proteins to be

$$S_u(V, q, N) = V(1-q)^N \quad (\text{S6})$$

We wish to estimate the critical number of proteins N_c required to cover the target volume; since the sequence space is discrete, we estimate N_c as the number of proteins for which there is $O(1)$ remaining uncovered sequence in the target volume. This yields

$$V(1-q)^{N_c} = 1 \quad (\text{S7})$$

which implies

$$N_c = \frac{\log(1/V)}{\log(1-q)} \quad (\text{S8})$$

The estimate (S8) appears to adequately describe the SNQ simulation data presented in the main text, as indicated by the scaling collapses shown in Fig. 3 of the main text. We expect the quality of the estimate to degrade, however, as the discrete nature of the sequence space becomes more important, i.e., as the number of sequences in the target volume $V(L, R)$ becomes small (of $O(1)$). Indeed, for the situation $R = 0$, where there is only one sequence in the target volume to be covered (namely the target sequence T), the estimate (S8) yields $N_c = 0$. For this case, however, we can independently estimate the number of randomly situated crosstalking sequences required to insure that the target sequence T is covered with probability $1/2$:

$$1 - (1-q)^{N_c^{R=0}} = 1/2 \quad (\text{S9})$$

implies

$$N_c^{R=0} = \log(1/2)/\log(1-q) \quad (\text{S10})$$

$$= \log(1/2)/\log(1-1/V_0) \quad (\text{S11})$$

The result (S8) assumes an alphabet size $q = 2$ (i.e., binary sequences). We can generalize the satisfiability bound in a straightforward manner, if we assume that binding of two sequences continues to be dictated by a maximal Hamming distance, i.e., two sequences s_1 and s_2 will bind if $H(s_1, s_2) \leq R$. In this case, the form of the bound (S8) remains unchanged, and we need simply redefine the relevant sequence volumes corresponding to an alphabet of size q :

$$V(L, R) = V(L, R, q) = \sum_{n=0}^R \binom{L}{n} (q-1)^n \quad (\text{S12})$$

$$V_0(L) = V_0(L, q) = q^L \quad (\text{S13})$$

In the case of reverse complement symmetric (RCS) sequences (e.g., for binding of protein to DNA in the regulation of gene transcription), the bound is reduced because each sequence in the target volume can be covered either by a ball centered within Hamming distance R of the sequence, or by a ball centered within distance R of the reverse complement of that sequence. This has the effect of doubling the coverage ratio q : $q \equiv 2V/V_0$. As a result,

$$N_c^{RCS} = \frac{\log(1/V)}{\log(1-2V/V_0)} \quad (\text{S14})$$

which is valid for $R < L/2$. For $R \geq L/2$, $N_c^{RCS} = 1$.

The main text alludes to a symmetric generalization of the SNQ that asks whether every protein in a collection is distinguishable, that is, whether there is a separate sequence niche for each of N proteins. While we do not have a general estimate for the critical number of proteins N_c for this problem, we can produce such an estimate for the special case of $R = 0$, where crosstalk occurs only if two sequences are exactly the same (no mismatches). In that limit, the question boils down to this: For binary sequences of length L , how many randomly chosen sequences must be chosen for there to be a probability of at least $1/2$ that two sequences are identical? This is just the classic “birthday problem” of probability theory, for a system where a “year” contains $V_0 = 2^L$ possible days (see, e.g., http://en.wikipedia.org/wiki/Birthday_problem). The probability $p(n)$ that two sequences out of n will match is:

$$p(n) = 1 - \frac{V_0!}{(V_0 - n)! V_0^n} \quad (\text{S15})$$

so, for a given sequence length L , we can find the number N_c for which this probability exceeds $1/2$ to arrive at an estimate for the $R = 0$ bound of the generalized SNQ.

In this light, the $R = 0$ case for the original SNQ (eq. (S11)) can be seen as a variant of the “my birthday problem”, which asks for the probability that someone in a group of N people will share *my* birthday. The probability of any crosstalk sequence matching the target sequence (in the original SNQ) is of course smaller than the

probability that any two crosstalk sequences will match each other (in the generalized SNQ). For $R > 0$, estimating the bound would seem to be a variant of the near-match birthday problem [44], but in higher dimensions.

S.2. Size of the largest solution cluster

Fig. 4(d) of the main text demonstrates that the size S_0 of the largest cluster (solid line) decreases roughly exponentially with crosstalk number N . From the geometric argument illustrated in Fig. 3(a) in the main text, we might expect

$$S_0 \sim (1 - q)^N \approx \exp(-qN) \quad \text{for small } q \quad (\text{S16})$$

where $q \equiv V(L, R)/V_0(L)$ as in eq. (S3). For $L = 16, R = 6$, $q \approx 0.23$, and a fit to the cluster size data in Fig. 4(d) reveals $S_0 \sim \exp(-0.29N)$. The exponential approximation to the power law in eq. (S16) would be more accurate for smaller q , but part of the discrepancy between the predicted and measured decay rate is due to the fact that the geometric argument only describes the elimination of viable sequences by crosstalk proteins, and not the fragmentation of clusters. Some of the decrease in S_0 is due to the latter effect.

S.3. Review of results from Zarrinpar, Park and Lim

We describe here in slightly more detail the experimental results of ref. [8]. Zarrinpar *et al.* investigated SH3-mediated signaling in yeast (*Saccharomyces cerevisiae*), probing in particular the signaling pathway involved in a high-osmolarity response, predicated on the interaction of the Sho1 protein (containing an SH3 domain) and the Pbs2 protein (with an exposed proline-rich, PXXP, peptide sequence). Experimentally, they created chimeric versions of the Sho1 protein, replacing the native SH3 domain with each of the other 26 SH3 domains found in yeast. (Three of the Sho1 chimeras were insoluble, however, so they could not be assayed *in vivo*.) They then sought to determine whether any of those domains could reconstitute the function of the high-osmolarity pathway, and found that none of the other yeast domains could so function. *In vitro* peptide binding assays also carried out revealed a similar lack of interaction from any but the Sho1-Pbs2 pair. When SH3 domains from 12 metazoan proteins were tested (both *in vivo* and *in vitro*), however, it was discovered that 6 of those were able to reconstitute the function of the high-osmolarity pathway. Their interpretation was that there has been an evolutionary selection against crosstalk in yeast, whereby domains and peptides have evolved such that the Pbs2 PXXP motif lies in a niche in sequence space where it is recognized by only the Sho1 SH3 domain, as is illustrated schematically in Fig. S.1(a). Since there has been no such selection pressure in other organisms, it was perhaps not

surprising that the Pbs2 motif overlaps with the recognition volumes of many of non-yeast SH3 proteins, as is illustrated in Fig. S.1(b).

Zarrinpar *et al.* also sought to characterize the nature of protein-protein interactions in the sequence space surrounding the wild-type Pbs2 motif, which they did by assaying a library of 19 single-base-pair missense mutations to the native yeast Pbs2 motif (leaving the core prolines of the PXXP motif unchanged). While some mutations resulted in increase affinity for Sho1, and some resulted in decreased affinity, all mutations resulted in an increased cross-reactivity with other yeast SH3 domains. This suggests that the wild-type Pbs2 is optimized not for affinity, but for discrimination among different SH3 domains.

S.4. Methods

To ascertain whether a given instance of the SNQ was satisfiable or not, I implemented the algorithm by Gramm *et al.* [23] (“Algorithm D” in [23], modified as described to treat the Distinguishing String Selection Problem). This is a recursive, backtracking algorithm in the style of Davis-Putnam(DP)-type methods used in the study of other NP-complete problems (e.g., k -SAT [45]). Algorithm D in [23] implements heuristics to prune the search tree, tailored to the Distinguishing String Selection Problem (DSSP). DP-type algorithms are known to be significantly slower in practice for k -SAT than other algorithms (e.g., WalkSAT [46] or survey propagation [30]), but have the advantage of being *complete*, i.e., able to determine whether any instance is satisfiable or not, given sufficient computer time. (Incomplete algorithms can typically find a solution if there is one, but are not guaranteed to stop if there is no solution.) For forays into a newly-identified NP-complete problem such as this, complete algorithms are a useful first step. For each SNQ instance, it was determined whether the instance was satisfiable, and how long it took to decide that question. Since DP-type methods are recursive, it is conventional to measure algorithm run times in units of number of calls to the recursive core, which is what we have done here.

The SNQ, as stated, applies to any set of sequences T and $\{C\}$. This paper has focused on random instances of the SNQ, where the relevant sequences are sampled uniformly at random from the set of all binary sequences of length L , with equal probabilities of 0 and 1 in the sequences T and $\{C\}$. Simulations of random instances of the SNQ were carried out, for various values of the relevant control parameters: the string length L , the Hamming radius R , and the number of crosstalk proteins N . Average satisfiability and algorithmic run time were computed from 100 random SNQ instances for each set of L , R , and N .

To explore the full solution space of SNQ instances, exhaustive examination was carried out. For each of the possible 2^L sequences, it was determined whether that se-

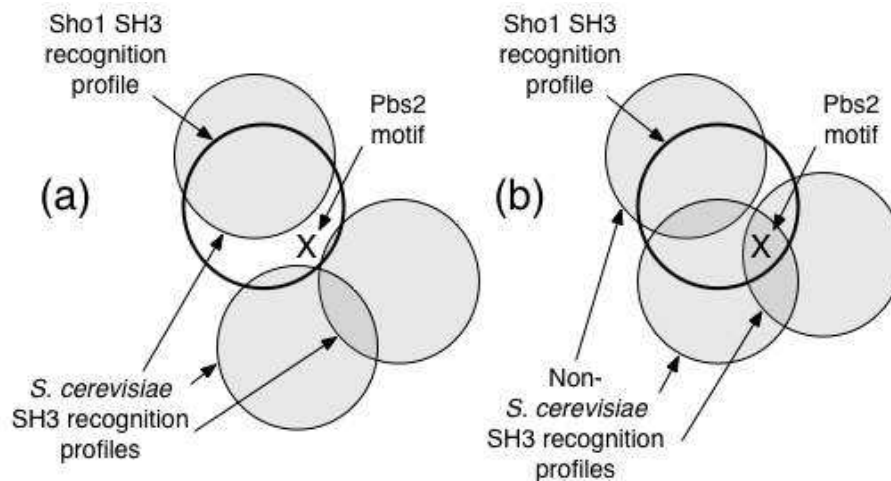


FIG. S.1: The interpretation offered by Zarrinpar, Park and Lim to describe (a) the lack of crosstalk among *S. cerevisiae* SH3 domains and (b) the presence of crosstalk among non-*S. cerevisiae* SH3 domains. [Adapted from [8].] (a) In *S. cerevisiae*, evolutionary selection against crosstalk has driven the proline-rich Pbs2 motif to a niche where it is recognized only by the Sho1 SH3 domain. (b) There is no such selection pressure in other organisms, so domains introduced from elsewhere can bind Pbs2.

quence satisfied the given SNQ. The set of valid solutions was assembled to form an undirected graph, whose nodes were SNQ solutions and whose edges joined nodes with sequences that differed by Hamming distance of 1, i.e., by 1 bit flip. The network analysis package NetworkX [networkx.lanl.gov] was used to compute connected components of the resulting graphs, and to generate layouts for visual display. This work motivated a contribution on my part to the NetworkX source code repository [networkx.lanl.gov/changeset/223], using tuples of index coordinates to label grid graphs, such as would be used to represent an L -dimensional hypercube. This representa-

tion is natural for graphs connecting nodes in sequence space. A spring force layout algorithm was used to generate the images in Figs. 4(a)-(c) in the main text, whereby connected nodes are attracted to each other to produce compact representations of connected components. As noted, however, the positions of the graph nodes in Figs. 4(a)-(c) have no intrinsic meaning, as all nodes are vertices on the L -dimensional hypercube. The problem of usefully visualizing complex network structures in high-dimensional sequence spaces is an ongoing challenge in computational biology.

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